
***Parvulastra exigua* in South Africa: one species or more?**

Robyn P. Payne

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Supervisors:

Prof. Charles Griffiths

Department of Biological Sciences, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

Dr Sophie von der Heyden

Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa



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Abstract

Parvulastra exigua is a widely distributed and prominent member of the temperate intertidal fauna in the southern hemisphere, occurring along the southern coastline of Africa, southeastern Australia and several oceanic islands. In South Africa, it is found in sympatry with the endemic *Parvulastra dyscrita* and the two are differentiated predominantly by gonopore placement. *P. exigua* gives rise to distinct lecithotrophic benthic larvae that hatch from sticky egg masses laid via oral gonopores. In contrast, *P. dyscrita* has aboral gonopores that release eggs into the water column, from which pelagic larvae hatch. Several recent studies have suggested that there is a cryptic species of *P. exigua* in South Africa, based on genetic evidence or the differential placement of the gonopores. A morphological, anatomical and genetic investigation was performed on a total collection of 346 *P. exigua* and 8 *P. dyscrita* specimens from the east and west coast of South Africa, with the hope of confirming whether cryptic species and/or *P. exigua* specimens with aboral gonopores are present in the population and determining if they correlate. Neither the cryptic species, nor *P. exigua* specimens with aboral gonopores were obtained. This study tentatively refutes the claim of the existence of aboral gonopores in the South African *P. exigua* population, and the distinction between *P. exigua* and *P. dyscrita* is confirmed, with features separating these two species clarified.

Key words: *Parvulastra dyscrita*, *Parvulastra exigua*, gonopore, cryptic species, *Patiriella*

1. Introduction

The dwarf cushion-star, *Parvulastra exigua* (Lamarck 1816), is a prominent member of the temperate intertidal fauna in the southern hemisphere (Hart et al. 2006). Occurring along the southern coastline of Africa (from Namibia to Mozambique), southeastern Australia and several oceanic islands (St. Helena, St. Paul, Amsterdam and Lord Howe) (Clark and Downey 1992), it's widespread distribution is attributed to the eastward rafting of individuals from Africa, via wood or macroalgae, during the Pleistocene (Waters and Roy 2004). As suggested by Barbosa et al. (2012), the establishment of founder populations may have been facilitated by the complex reproductive methods observed in this species, which have largely been studied in Australian populations (Hart et al. 2006).

P. exigua is an ovipositor with continuous oogenesis. Spawning predominantly from August to October (Lawson-Kerr and Anderson 1978; Byrne 1992), this species gives rise to distinct lecithotrophic benthic larvae (Byrne and Anderson 1994; Figure 1) that hatch from sticky egg masses laid via oral gonopores (Lawson-Kerr and Anderson 1978). Protandry-like hermaphroditism is often exhibited (Byrne 1992), while simultaneous hermaphrodites (usually of intermediate size) can also occur (Byrne and Anderson 1994). In addition, females may self-fertilize by producing a small quantity of sperm in their gonads (Byrne and Anderson 1994). However, Barbosa et al. (2012) suggest that the primary mode of reproduction in this species is outcrossing via direct sperm release onto communal egg masses (supported in South Africa; personal observation), in a form of pseudocopulation (Byrne 1992). Several key features of *P. exigua*'s reproduction (including gametic incompatibility) and development act to isolate it from other sympatric cushion-star species, such as *Patiriella calcar* and *Patiriella gunnii* in Australia (Byrne and Anderson 1994).

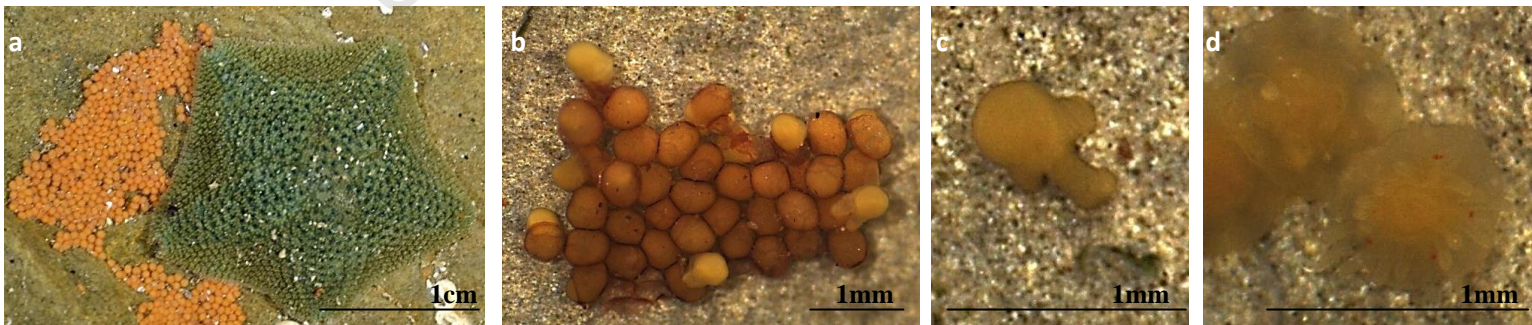


Figure 1: (a) Adult *Parvulastra exigua* laying sticky eggs via oral gonopores onto the underside of a rock (© Charles Griffiths), (b) a close up of the benthic egg mass with emergent larvae. (c) Enlarged view of lecithotrophic larva and at a later stage (d) in which tube feet and red eyespots can be seen.

In South Africa, *P. exigua* occurs in sympatry with the endemic granular cushion-star, *Parvulastra dyscrita* (Clark 1923) and the two have a somewhat intertwined taxonomic history. *P. exigua* was first described as *Asterias exigua* by Lamarck in 1816, while Perrier (1875) found this species to be conspecific with *Asterina exigua* Lamarck, *Asterina kraussii* Gray and *Asteriscus pentagonus* Müller and Troschel. Whitelegge (1889) was the first to note the oral gonopore placement of *Asterina exigua* with Mortensen (1921) confirming this. In 1913, Verrill moved several species, including *Asterina exigua*, into the new genus *Patiriella*, but this change was often ignored. A new species (*Parvulastra dyscrita*) with aboral gonopores, *Asterina dyscrita* was described by Clark in 1923, with the suggestion that it may only be a variety of *Patiriella exigua*. Mortensen (1933) then placed *Asterina dyscrita* in synonymy with *Patiriella exigua*. Dartnall (1971) reviewed *Asterina* (*Patiriella*) *exigua*, which consisted of both *Parvulastra exigua* and *Parvulastra dyscrita* specimens at the time and recognised them as two different species according to gonopore placement. Clark (1974) recognised the latter as a conspecific of *Asterina dyscrita* and moved it to the genus *Patiriella* due to its morphological similarity to *Patiriella exigua*. Recently, both species were moved to the new genus *Parvulastra* (O' Loughlin and Waters 2004) with *Patiriella vivipara*, *Patiriella parvivipara* and *Patiriella calcarata*. This genus is distinguished from *Patiriella* based on ray width, ray plate alignment and is supported by previous molecular studies conducted by Waters et al. (2004)

Today, *P. exigua* and *P. dyscrita* are still differentiated predominantly via gonopore position (Table 1), with the latter occurring in low densities, subtidally along the south/east coast between False Bay and East London (33° 4'S, 27°50'E) (Branch et al. 2010). In contrast to *P. exigua*, *P. dyscrita* releases eggs into the water column via aboral gonopores, where they hatch into planktonic larvae. While the larger *P. dyscrita* often demonstrates a mottled colouration with shades of pale pink, white and brown, *P. exigua* demonstrates high morphological variability in South Africa (Figure 2), with two colour morphs demonstrating an allopatric distribution and separated by a narrow hybrid zone (Dunbar 2007) approximately 0.5km from Cape Point (34°24'S, 18°29'E). On the west coast, *P. exigua* are of a uniform khaki green colour (similar to Australian populations), while often brightly coloured, variegated individuals occur on the east coast (Branch et al. 2010).

Dunbar (2007) found the colour morphs to demonstrate some degree of ecological divergence with regards to tidal zone and microhabitat, with the mottled morph predominantly found in the mid to low tidal zone on crustose coralline algae, while the green morph occupies the high tidal zone on the west coast. This breaks down in the hybrid zone, where green and mottled *P. exigua* colour morphs, as well as intermediates (both phenotypically and ecologically) can be found together and show no tidal zone or microhabitat preference. In addition, when these morphs occur together along the east coast (with low densities of green individuals), the green morphs do not demonstrate tidal zone preference but often occur amongst fleshy algae and fauna.



Figure 2: Abactinal surface view of (a) the *Parvulastra exigua* mottled colour morph found on the east coast of South Africa, (b) the *Parvulastra exigua* green colour morph found predominantly on the west coast of South Africa and (c) *Parvulastra dyscrita* (© Charles Griffiths). All scale bars represent 1cm.

These colour morphs, which are possibly a response to a clinal environmental gradient (such as temperature), were shown by Dunbar (2007) to be the same species, with spatial separation maintaining the observed colour polymorphism. East coast individuals were found to be genetically twice as diverse as west coast individuals, while the intermediate colour morph was found to be twice as diverse as both the green and mottled colour morphs (Dunbar 2007). It was also found that specimens believed to be *P. exigua* did not form a monophyletic clade (with the same outcome for *P. dyscrita*) which would disqualify them from being classed as separate species.

Additionally, Dunbar (2007) identified a highly divergent haplotype in two Kommetjie specimens, suggesting that a cryptic species of *P. exigua* was present in South Africa. In 2006, Hart et al. externally surveyed the gonopore position in *P. exigua* museum collections from South Africa, southern Australia and several islands (St. Helena, Amsterdam, St. Paul and Kerguelen) for evidence that some *P. exigua* populations include cryptic species with a different mode of reproduction. Overall, 33% (21% in South Africa) of the *P. exigua* specimens were found to have aboral gonopores, with such individuals occurring predominantly in South Africa and the St. Paul, Amsterdam and St. Helena islands. Mitochondrial DNA sequence data from the study by Waters and Roy (2004) was also considered, with Hart et al. (2006) tentatively suggesting that a cryptic species of *P. exigua* (or more) exist in South Africa, probably with aboral gonopores.

Thus, the aim of this study was to sample *P. exigua* populations along both the west and east coast of South Africa with the hope of confirming whether cryptic species and/or *P. exigua* specimens with aboral gonopores are present in the population and determining if they correlate. This is done by examining the morphology and anatomy of each collected specimen, as well as carrying out genetic analyses. It is expected that any cryptic species of *P. exigua* will have aboral gonopores.

Table 1: Characteristics that distinguish *Parvulastra exigua* from *Parvulastra dyscrita*, based on published literature.

Characteristic	<i>Parvulastra exigua</i>	<i>Parvulastra dyscrita</i>	Source
Size	Small, up to 20mm.	Larger, up to 40mm.	Branch et al. (2010)
Abactinal surface colouration	Dull khaki-green on the west coast of South Africa. Variegated (often geometrical) patterns on the east coast, including most colour combinations.	Mottled shades of pale pink, white, purple and maroon.	Clark (1923); Clark and Courtman-Stock(1976); Branch et al. (2010)
Actinal surface colouration	Consistently blue-green.	Not consistently blue-green.	Dartnall (1971);Clark (1974)
Abactinal surface spinulation	Fine, short columnar.	Coarse, granuliform globose.	Clark (1923); O' Loughlin and Waters (2004); Branch et al. (2010)
Abactinal surface texture	Clusters of spines.	Evenly granular.	Branch et al. (2010)
Papulae	Large.	Small, numerous.	Clark (1923); O' Loughlin and Waters (2004)
Actinal intermediate plate spinulation	Each plate with only one or two spines, with the latter occurring more frequently distally.	Many plates with two spines each.	Clark (1923); Dartnall (1971); O' Loughlin and Waters (2004)
Adradial actinal spines	Absent.	Present.	O' Loughlin and Waters (2004)
Furrow/ Ambulacral spines	Two (sometimes three) slender, short spines.	Two (sometimes three) slender, short spines.	Clark 1923; Dartnall (1971); O' Loughlin and Waters (2004)
Subambulacral spines	Tall, thick, pointed spine on each adambulacral plate.	Large, blunt, truncate spine on each adambulacral plate.	Clark (1923); Dartnall (1971); O' Loughlin and Waters (2004)
Oral plates	Two tall oral spines per plate, with three-five marginal spines.	Two tall oral spines per plate, with six or seven marginal spines.	Clark (1923); O' Loughlin and Waters (2004)
Gonopore position	Oral ,two in each interradius, separated from oral plates by approximately three actinal plates.	Aboral.	Dartnall (1971);Clark and Courtman-Stock(1976) O' Loughlin and Waters (2004)

2. Methods

2.1. Specimen collection

Approximately 90 starfish were collected from intertidal rocky shores at Kalk Bay, Hermanus, Kommetjie and Britannia Bay during low tide (Figure 3; Table 2). At each site, the rocky shore was visually divided into three vertical intertidal zones: high-, mid- and low-shore, with 30 specimens being collected from each. An exception was Britannia Bay, where few starfish could be found in the lowest zone. In addition, several specimens were collected from around the coast to enhance the *P. dyscrita* sample size, and obtain any peculiar *P. exigua*.

Table 2: Number of starfish collected per sampling location, with geographic coordinates. Brackets indicate the number of *Parvulastra dyscrita* collected.

	Location	Coordinates		High	Medium	Low	Sand	Total
West Coast	1. Britannia Bay	32°43'S	17°56'E	33	31	2	-	66
	2. Langebaan Lagoon	33°6'S	18°01'E	-	-	-	9	9
	3. Kommetjie	34°8'S	18°19'E	33	30	33	-	96
East Coast	4. Kalk Bay	34°7'S	18°26'E	30	30	34(6)	-	94
	5. Strandfontein	34°4'S	18°34'E	-	-	2 (1)	-	2
	6. Macassar	34°4'S	18°44'E	-	-	(1)	-	1
	7. Hermanus	34°24'S	19°15'E	33	32	30	-	95
								363



2.2. Morphology and anatomy

The oral and aboral surface of each specimen was photographed after collection in order to document colour. After preservation in 70% ethanol, several qualitative and quantitative characteristics, based predominantly on previous taxonomic descriptions from both known species, were recorded (Figure 4; Table 3). In addition, marginal plate spines, tube feet and the madreporite exterior were examined per specimen, but these characteristics were excluded from further analyses, as they demonstrated no difference from one specimen to the next. Apart from documenting colour morph type, abactinal and actinal surface colouration as well as R and r values were also eliminated due to the high variability observed and possibility of skewing the analyses respectively.

Figure 3: Map depicting sampling locations along the South African coast. Numbers correspond to locations in Table 2.

Table 3: Starfish characteristics examined per specimen and used in further multivariate analyses. Abbreviations in brackets correspond to Figure 4. All measurements were carried out using a digital caliper to two decimal places, while observations were done with the aid of a microscope.

	Characteristic	Technique
Quantitative	R/r	Expression of body proportion. R-greater radius measured along the ambulacral groove. r-smaller, interambulacral radius.(Dartnall 1971) Both were measured along three non-deformed arms per specimen and then averaged.
	Peristomial membrane diameter (pmd)	Used as an expression of body size. Measured twice per specimen and averaged.
	Madreporite diameter (md)	Measured twice per specimen and averaged.
	Papulae diameter (pd)	Five papula diameters were measured per specimen and averaged.
	Oral plate teeth (opt)	Number of oral plate teeth documented.
	Oral plate spinulation	Number of spines per oral plate documented.
	Oral marginal plate spinulation (omps)	Number of oral marginal spines noted.
Qualitative	Colour morph	Each specimen was described as either a green or mottled morph.
	Abactinal surface spinulation	Specimens were described as having either fine, short columnar or coarse, granuliform globose spinelets.
	Abactinal surface texture	Described as either clusters of spinelets or an evenly granular surface texture.
	Adradial actinal spinulation (aas)	Documented as absent or present. In the case of the latter, it was further documented whether these spines occurred in more, or less than three arms.
	Furrow/ Ambulacral spinulation	Specimens classified according to the relative number of plates with one spine, as well as the presence or abundance of three spines per plate.
	Actinal intermediate plate spinulation	Specimens classified according to the relative number of plates with one or two spines, starting position of the plates with two spines and the presence of plates with three spines.
	Visible gonopore position	Documented as having either oral gonopores or none (aboral gonopores are difficult to observe).
	Gonopore position	Specimens were dissected to determine gonad placement and definitively document gonopore position.

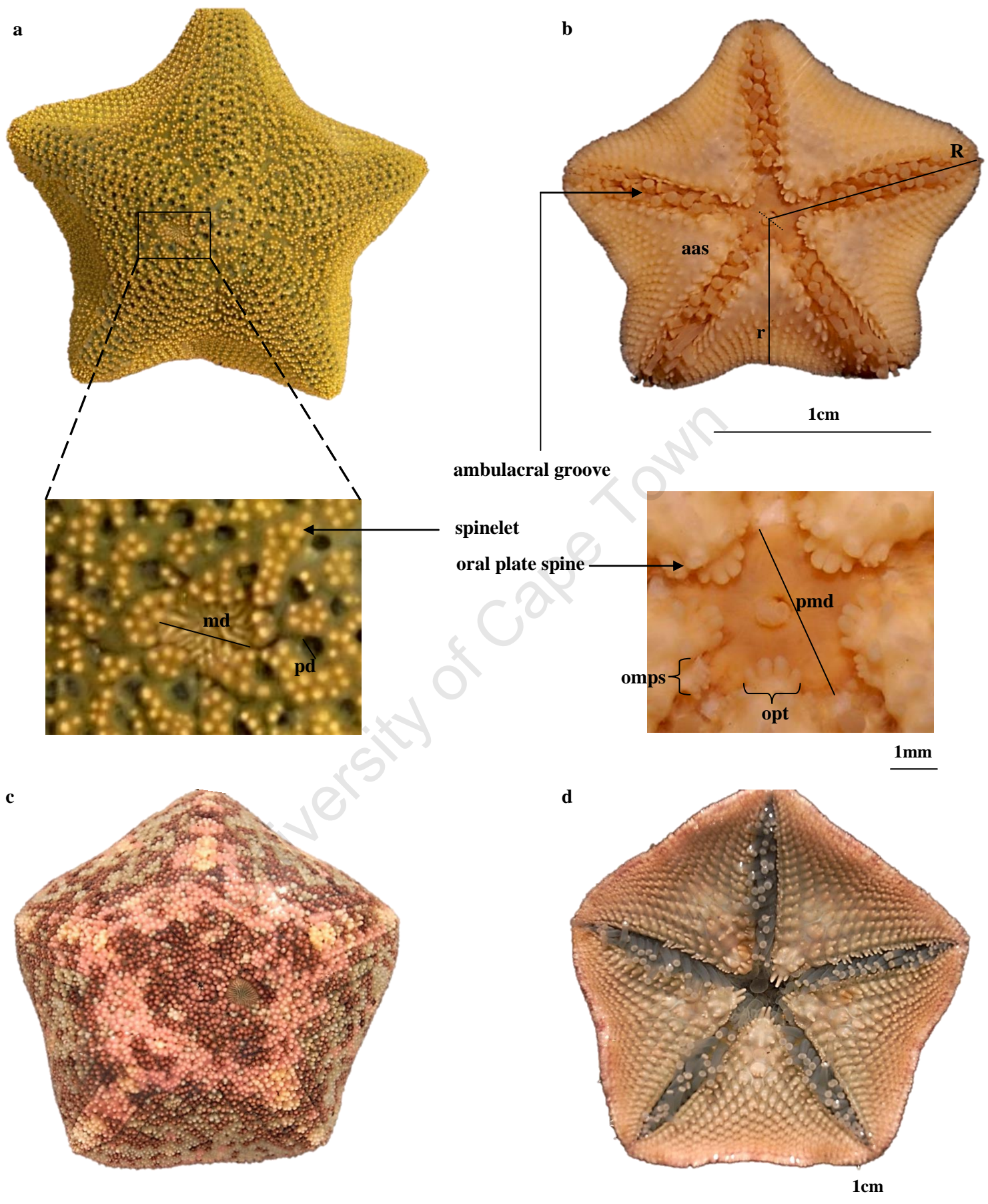


Figure 4: Abactinal (a) and actinal (b) view of *Parvulastra exigua*, with equivalent views of *Parvulastra dyscrita* (c, d) for comparison. Abbreviations follow Table 3.

2.2.1. Statistical analyses

Overall, 354 specimens were included in the analyses, with 9 being excluded due to degradation and the subsequent ambiguity in documenting characteristics.

Multivariate analyses were performed on unstandardised and untransformed characteristic data using PRIMER v.6.1.5 (Plymouth Routines in Multivariate Ecological Research; Clarke and Gorley 2006). Non-metric multidimensional scaling (MDS) ordinations, based on a resemblance matrix generated from Bray-Curtis similarities, were used to visually assess specimen similarity. Additionally, these ordinations were utilized to identify outlying individuals which were included in the genetic analyses. Six arbitrary groups were defined (Figure 5; Table 4) with each comprising seven outlying individuals that were believed to possibly represent the cryptic species of *P. exigua*. A seventh group, consisting of seven *P. dyscrita*, was included for comparison.

The one-way ANOSIM (analysis of similarity) routine was performed in order to determine whether possible specimen groupings are associated with location, tidal zone, coast or any of the documented characteristics, with the significance of the statistical tests assigned at the 5% level. Thereafter, SIMPER (similarity percentage analysis) was used to determine the characteristics that contribute to at least 90% of the difference between divergent cluster groups.

Table 4: Specimen groups selected for genetic analyses. Each group comprises of seven outlying individuals identified on an MDS.

Group	Description
1. Mottled	Individuals found on the east coast with variegated colouration.
2. Green	Individuals found on the west coast with green colouration.
3. Orange	Individuals found on the west coast with orange colouration.
4. Two oral plate teeth	Individuals with two oral plate teeth, as opposed to the common four oral plate teeth observed in the majority of specimens collected.
5. Langebaan	Individuals from a sand flat with deep aboral dimples and peculiar abactinal surface spinulation. Some also appear non-pentagonal.
6. Peculiar	Individuals with atypical colouration, shape, size e.t.c.
7. <i>P. dyscrita</i>	Included for comparison.



Figure 5: Four specimens, from each defined group, which were used in the genetic analyses. Numbers to the left correspond to groups seen in Table 4. All specimens shown are approximately life size.

2.3. Genetics

2.3.1. DNA extraction, PCR, sequencing and alignment

Overall, 49 specimens were selected for the genetic analyses. Each specimen was dissected, and approximately 25 mg piece of arm was removed and placed in a solution of 25µl Proteinase K and 180µl of the manufacturers Buffer T1. After vortexing, the sample was incubated for 1-3 hours at 56°C until lysis was obtained. Thereafter, genomic DNA was extracted via the use of a NucleoSpin® Tissue kit (Macherey-Nagel) and stored at -20°C.

The Polymerase Chain Reaction (PCR) took place in a MultiGene thermal cycler (Labnet), amplifying a segment of the mitochondrial cytochrome *c* oxidase I (COI) gene (592-984bp) using a combination of the universal invertebrate primers (Folmer et al. 1994), LC01490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') as well as those designed by Mertens (2012) specifically for *P. exigua*: Pexig_F1 (5'-CTTTCCCACGAATGAACAAYATGAGC-3') and Pexig_R1 (5'-CCGAGGGCTCATAGGAGAGGAGTGTC-3'). All amplifications were performed in 25µl reactions containing 13.4µl milliQ water, 2.5µl Supertherm 10x polymerase buffer, 2.5µl dNTPs, 2µl MgCl₂, 1.25µl each primer and 0.1µl *Taq* polymerase. DNA dilutions (with milliQ water), primers used and the volume of DNA added differed according to specimen (Table 1A; Appendix). The optimal cycling parameters are given in Table 5. Negative controls were included for each reaction.

To determine if amplifications were successful, 5µl of each PCR product was loaded onto 1% agarose gels after being combined with 1µl loading dye. When confirmed, an ABI-3100 automated sequencer (Applied Biosystems) and Big Dye terminators were used to generate sequences. Clear reverse sequences were obtained for 44 of the 49 specimens, which were then edited and aligned manually using Geneious v.6.1.6. (Biomatters Ltd. 2013).

2.3.2. Genetic analyses

Geneious v.6.1.6. was used to build an unrooted neighbor-joining tree with bootstrap support from consensus sequences that had a final length of 345bp. These sequences were also imported into TCS v.1.21. (Clement et al., 2000) in order to generate a parsimony haplotype network template that was then enhanced using PowerPoint™ (Microsoft). For the latter analysis, a 95% plausible connection limit was used.

Table 5: Optimal PCR cycling parameters. Number of cycles were dependent on primers and DNA dilution used (see Table 1A; Appendix), which differed according to specimen.

Process	Temperature (°C)	Time (min)	No. of cycles
Initial denaturation	94	3	1
Denaturation	94	0.5	35-38
Annealing	45	0.75	35-38
Extension	72	0.75	35-38
Final extension	72	10	1

3. Results

3.1. Morphology and anatomy

3.1.1 General comments

Of the 354 specimens included in the analysis, 8 were identified as *P. dyscrita* through the careful consideration of all documented characteristics. *P. exigua* specimens collected from Kommetjie and Britannia Bay (west coast sampling sites) exhibited a variety of uniform abactinal surface colouration, such as: pale green, olive green, orange, brown and blue, while those from Hermanus and Kalk Bay (east coast sampling sites) were all mottled morphs, generally of darker colouration. No green morphs were sampled from the east coast collection sites and neither were intermediates. Individuals collected from Langebaan Lagoon were dark green, with some exhibiting an orange shoulder. In contrast to the colouration variability displayed by *P. exigua*, all *P. dyscrita* specimens displayed an abactinal surface with muted shades of pink, white, brown and turquoise. Actinal colouration was highly variable between and among sampling site specimens in *P. exigua*, while *P. dyscrita* individuals were often of a bluish-yellow colour orally.

The visual gonopore position was not visible in any *P. dyscrita* specimens, and once dissected it was clear that all individuals regarded as this species had aboral gonopores. A visual oral gonopore position could be seen in some preserved *P. exigua* specimens, while no gonopores could be seen in others. In the case of those collected from Langebaan Lagoon, all demonstrated large aboral dimples. However, when dissected, all *P. exigua* specimens displayed oral gonopores.

In addition to the difference in gonopore position, *P. exigua* and *P. dyscrita* differ with regards to several other quantitative and qualitative characteristics (Table 6). Generally, *P. dyscrita* is larger, with more spinulation.

3.1.2. Multivariate analyses

Two clear clusters are evident in Figure 6, with the specimens differing significantly according to all characteristics defined (Table 7), except R/r which is only marginally significant. However, the gonopore position, abactinal surface texture (both Figure 7a), abactinal surface spinulation (Figure 7b) and oral plate teeth (Figure 7c) play a predominant role in this configuration. This is consistent with the previously mentioned differences between *P. exigua* and *P. dyscrita*, and is also supported by the SIMPER results (Table 8) with the two clusters having an average dissimilarity of 35.96%.

Although the specimens differ significantly according to location ($R=0.225$, $p=0.001$), intertidal zone ($R=0.053$, $p=0.001$) and the coastline from which they were obtained ($R=0.187$; $p=0.001$), these factors play a rather small role in structuring the MDS (Figure 6) when compared to the other documented characteristics (Table 7).

Table 6: Overall characteristic summary of *Parvulastra exigua* and *Parvulastra dyscrita*, demonstrating the differences between the species.

	Characteristic	<i>Parvulastra exigua</i>	<i>Parvulastra dyscrita</i>
Quantitative	R/r	1.07-1.83	1.16-1.45
	Peristomial membrane diameter (mm)	1.26-6.10	4.70-7.15
	Madreporite diameter (mm)	0.19-2.92	2.27-4.62
	Papulae diameter (mm)	0.07-0.28	0.13-0.26
	Oral plate teeth	Four, two or variable.	Four or more, often variable.
	Oral plate spinulation	Two tall oral spines per plate, often consisting of two spines in the place of one.	Two tall oral spines per plate, often consisting of ‘bunches’ of spines in the place of one.
	Oral marginal plate spinulation	Three or four spines per plate, or a combination of the two.	Five or six spines per plate, or a combination of the two.
Qualitative	Colour morph	green/mottled	mottled
	Abactinal surface spinulation	short columnar	granuliform globose
	Abactinal surface texture	clusters of spines	evenly granular
	Adradial actinal spinulation	often present	absent
	Furrow/ Ambulacral spinulation	Two (often three) slender, short spines.	Two (often three) slender, short spines.
	Actinal intermediate plate spinulation	Each plate with only one or two spines, with the latter occurring more frequently distally.	Many plates with two spines each, some with three.
	Visible gonopore position	oral or none	none
	Gonopore position	oral	aboral

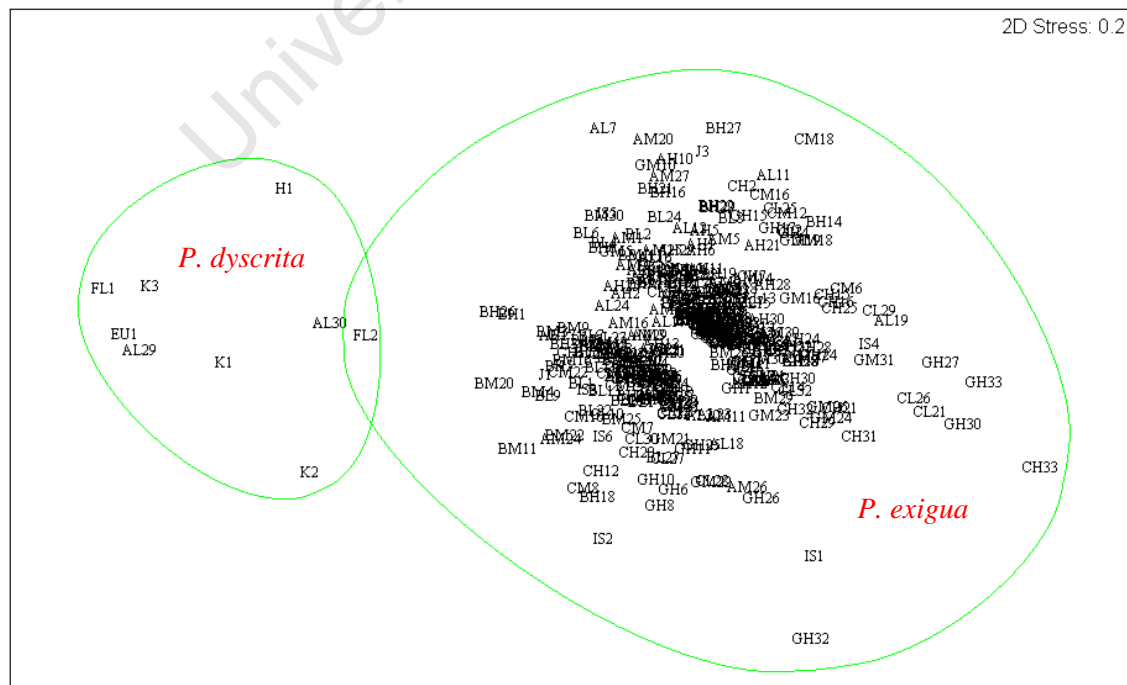


Figure 6: Non-metric MDS ordination of all specimens analysed, displaying two clear clusters. Circles indicate 70% similarity.

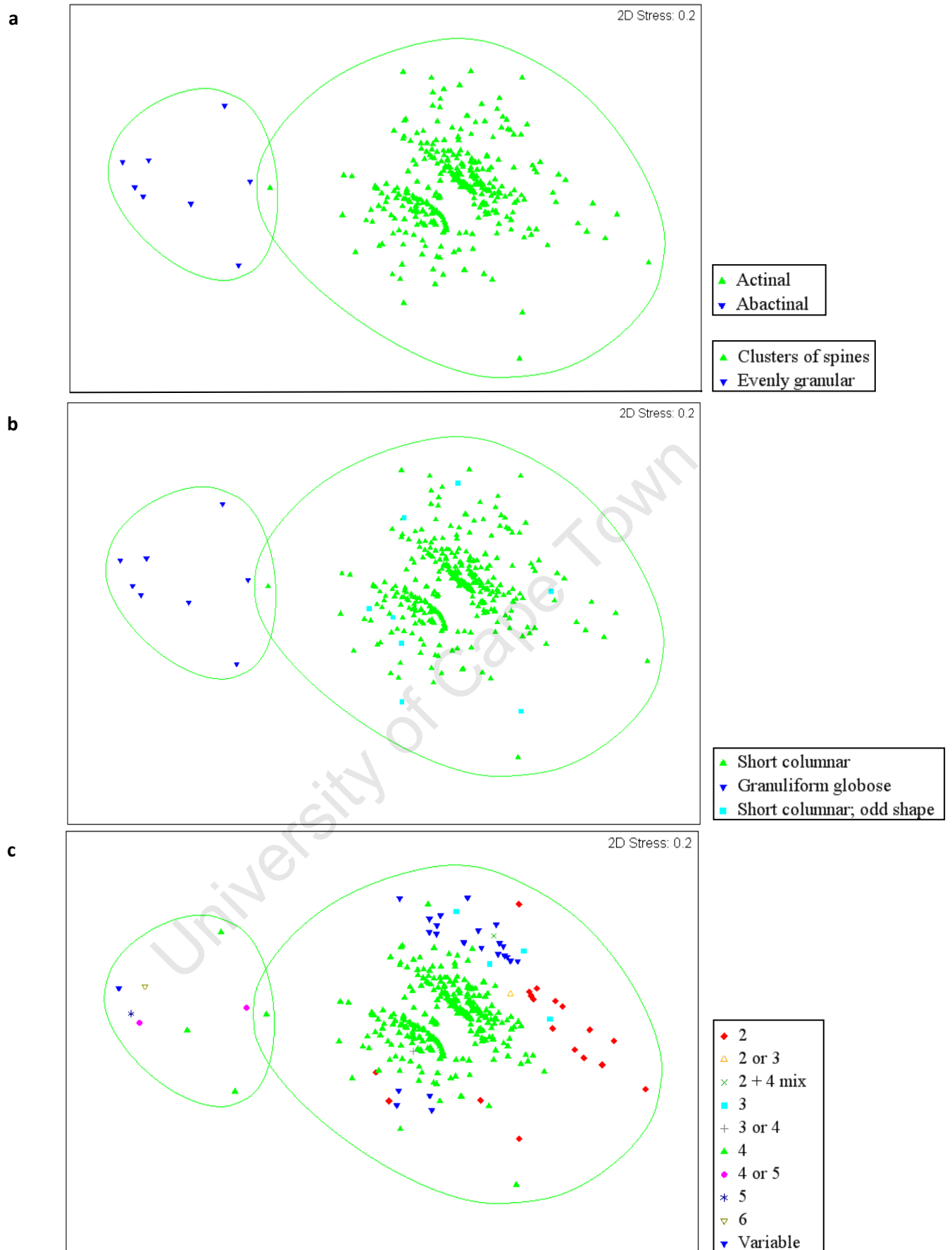


Figure 7: Non-metric MDS ordinations of all specimens according to (a) gonopore position and abactinal surface texture, (b) abactinal surface spinulation and (c) oral plate teeth .Circles indicates 70% similarity.

Table 7: ANOSIM results - R test statistic and significance per defined characteristic for all specimens analysed.

	Characteristic	R	p
Quantitative	R/r	0.023	0.051
	Peristomial membrane diameter	0.162	0.001
	Madreporite diameter	0.136	0.001
	Papulae diameter	0.066	0.001
	Oral plate teeth	0.682	0.001
	Oral plate spinulation	0.238	0.001
	Oral marginal plate spinulation	0.373	0.001
Qualitative	Colour morph	0.187	0.001
	Abactinal surface spinulation	0.757	0.001
	Abactinal surface texture	0.981	0.001
	Adradial actinal spinulation	0.364	0.001
	Furrow/ Ambulacral spinulation	0.411	0.001
	Actinal intermediate plate spinulation	0.503	0.001
	Visible gonopore position	0.291	0.001
	Gonopore position	0.981	0.001

Table 8 supports the differences between *P. exigua* and *P. dyscrita* seen in Table 6, with spinulation playing a major role (57.55%) in the delineation of these species.

Table 8: SIMPER results - percentage contribution of each characteristic that overall contribute to at least 90% of the difference between the divergent clusters.

Characteristic	% Contribution
Oral plate spinulation	27.2
Actinal intermediate plate spinulation	12.42
Oral marginal plate spinulation	9.04
Furrow/ Ambulacral spinulation	8.89
Peristomial membrane diameter	7.95
Madreporite diameter	7.06
Oral plate teeth	6.76
Adradial actinal spinulation	5.43
Abactinal surface texture	3.53
Gonopore position	3.53

Although the *P. exigua* specimens still differ significantly according to all characteristics defined (Table 9), this is to a much lesser extent. Oral plate teeth (Figure 9a) and abactinal surface spinulation (Figure 9b) are the largest contributors to the structure seen. However, there seems to be no morphological separation between *P. exigua* specimens that is great enough to indicate the presence of a cryptic species.

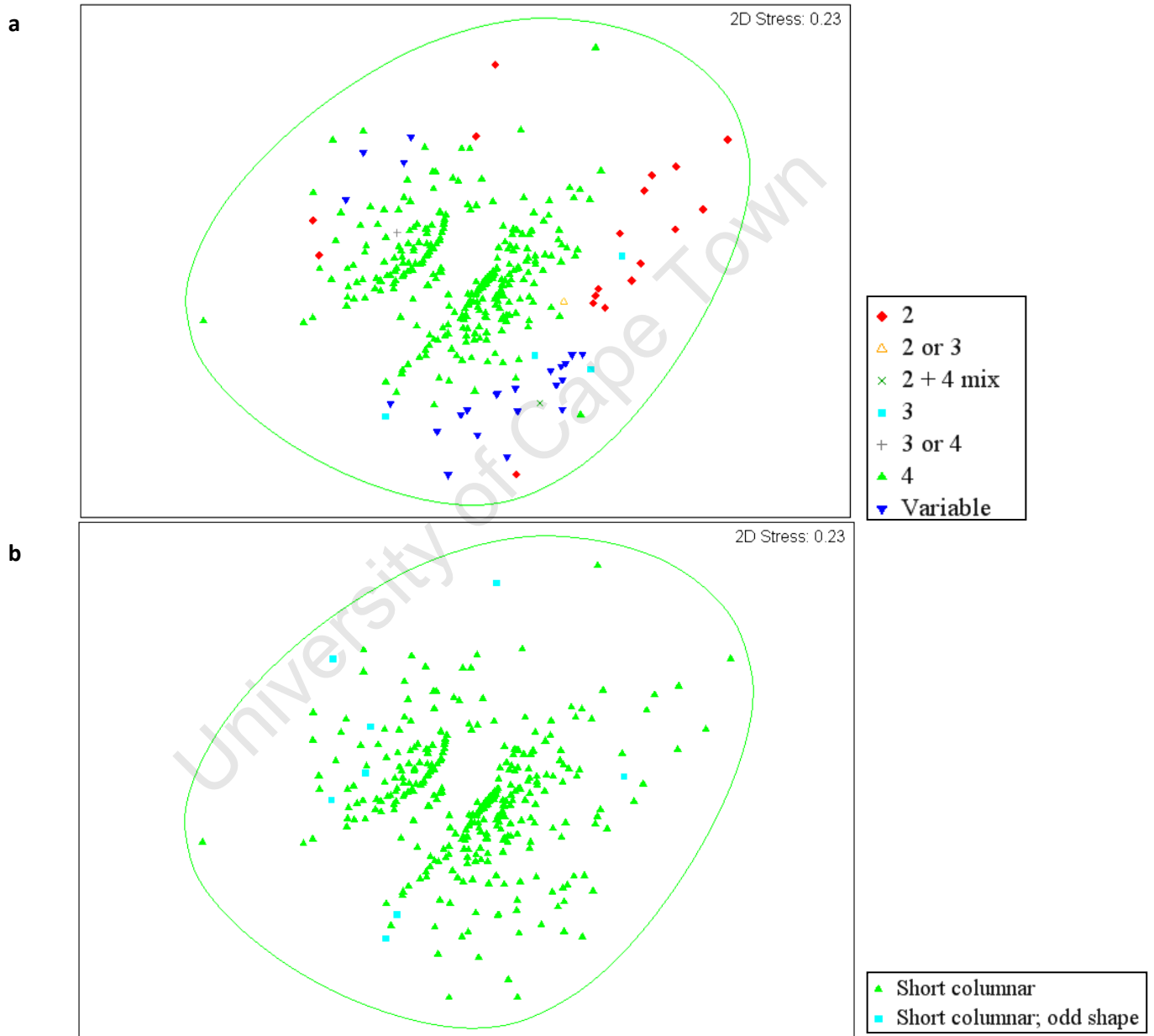


Figure 9: Non-metric MDS ordinations of all *P. exigua* specimens according to (a) oral plate teeth and (b) abactinal surface spinulation. Circle indicates 70% similarity.

3.2. Genetics

The unrooted neighbor joining tree (Figure 10) demonstrates a clear separation of the sequences into two clans with high bootstrap support. The clan to the left consists of seven sequence that are attributed to the species *P. dyscrita*, while all the *P. exigua* sequences fall into the clan on the right. The latter demonstrates little genetic distance, with no structure according to location, tidal height or specimen group.

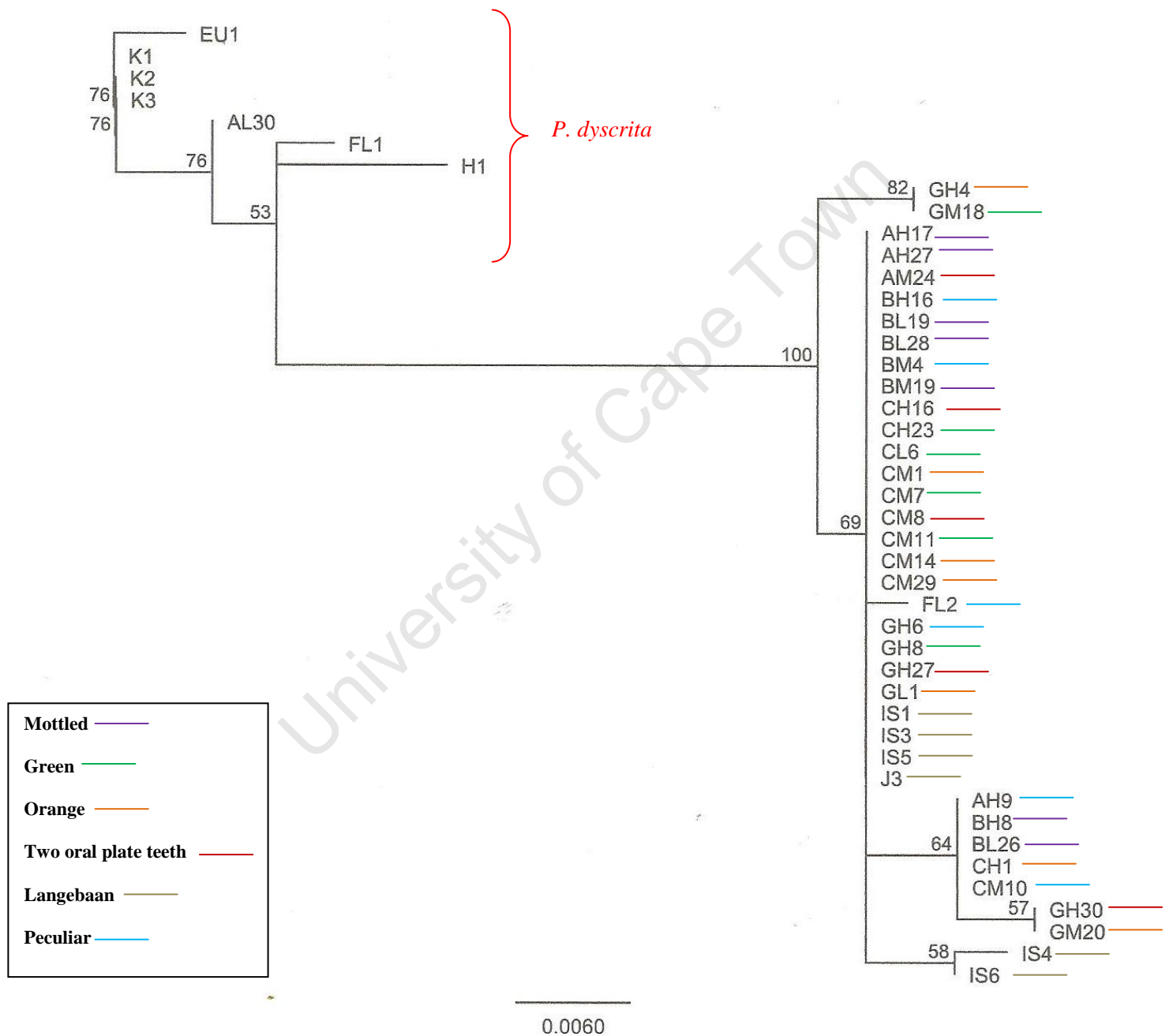


Figure 10: Unrooted Neighbor Joining Tree based on 44 COI sequences, where colour denotes specimen group. Level of support indicated by bootstrap values.

Two haplotype networks separated out from each other at the 95% connection limit (Figure 11), suggesting that the sequence alignment included two species. This is confirmed by the divergent clusters of *P. exigua* and *P. dyscrita* that separated out in Figure 6 based on morphology and anatomy, and the unrooted neighbor joining tree (Figure 10) that demonstrated a genetic separation of these two species.

The haplotype network for *P. exigua* (Figure 11a) is dominated by two main haplotypes which are well distributed across the sampling locations, with three unique haplotypes found along the west coast. The connections between the haplotypes indicate that they are genetically very similar (at the maximum five mutational steps distance), which is confirmed by the lack of morphological and molecular separation into clusters, as seen in Figure 8 and Figure 10 respectively.

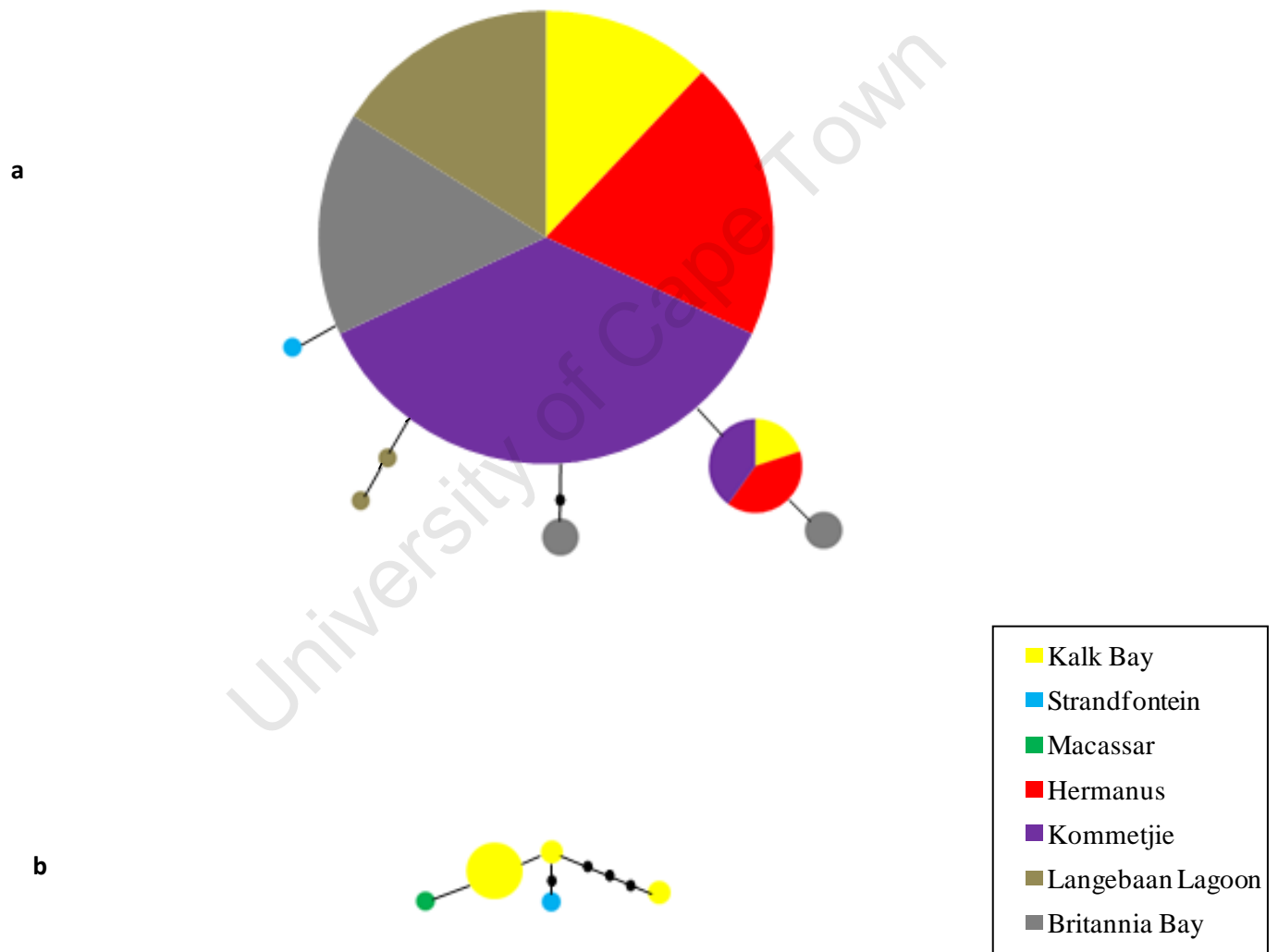


Figure 11: Parsimony haplotype networks for (a) the 37 *Parvulastra exigua* and (b) the 7 *Parvulastra dyscrita* specimens collected in this study. Circle size relates to the frequency of each haplotype, with colour indicating origin of the individuals. Smallest circles represent one individual and one haplotype. Extinct or not sampled haplotypes are marked by a black dot and each line represents one mutational step.

4. Discussion

The morphological, anatomical and genetic investigation into the possibility of cryptic species and/or aboral gonopores (with an expected correlation) in the South African *P. exigua* population yielded no cryptic species, nor any *P. exigua* specimens with aboral gonopores.

All *P. exigua* specimens collected exhibited oral gonopores, with only slight morphological differences in oral plate teeth and abactinal surface spinulation, but no separation great enough to indicate the presence of a cryptic species. This was confirmed by molecular analyses, with a clan of *P. exigua* sequences demonstrating little genetic difference, with no structure according to location, tidal height or specimen group. In addition, *P. exigua* haplotypes were genetically very similar. Mertens (2012) also conducted genetic analyses on 177 *P. exigua* specimens collected along the west coast of South Africa (from Kommetjie to Port Nolloth (29°18'S, 16°52'E)) and did not find signals of cryptic species. All of this evidence suggests that if there is a cryptic species, it occurs in very low numbers, possibly in a very narrow geographic range. The fact that it was of intermediate morph colouration, may suggest that it only occurs in the hybrid zone, 0.5 km from Cape Point.

Such results support Dunbar's (2007) conclusion that different colour morphs in South Africa were all simple variants of the same *P. exigua* species, and this current study highlights how morphologically variable this species is in South Africa.

4.1. The possibility of aboral gonopores in the South African *P. exigua* population

After no *P. exigua* specimens were found to have aboral gonopores, a further 200 *P. exigua* individuals were collected from Mouille Point (33°54'S, 18°24'E), a location where museum specimens with supposed aboral gonopores had been collected previously, and were examined by Hart et al. (2006). These specimens, once dissected, were also found to have oral gonopores. On enquiry, it was determined that Hart et al. (2006) only examined museum specimens externally (Michael Hart and Maria Byrne, personal communication) and it is suggested that they mistook abactinal dimples (or the lack of visible oral gonopores) for aboral gonopores. This proposal is supported by the fact that *P. dyscrita*, a species with aboral gonopores, has no easily identifiable external gonopore position, causing one to question how you would decipher gonopore position in this case, without dissecting the animal. A similar situation was faced with regards to the few *P. exigua* specimens examined that displayed no oral gonopores and had to be dissected in order to reveal that the gonads were orally directed. Finally, Langebaan Lagoon *P. exigua* specimens have deep abactinal grooves, with some lacking visible oral gonopores and unless dissected, could easily mislead an examiner into believing that they have aboral gonopores. It is also important to note that Waters and Roy (2004) did not include *P. dyscrita* in their molecular analysis and so some of the samples included in their analysis that depicts cryptic diversity in South Africa, could include *P. dyscrita* misidentified as *P. exigua* (Michael Hart, personal communication)

Thus, this study tentatively suggests that *P. exigua* specimens in South Africa with aboral gonopores do not exist.

4.2. *P. exigua* and *P. dyscrita*; sympatric species in South Africa

P. dyscrita and *P. exigua* show a clear separation based on morphology, anatomy and genetics, unambiguously revealing them as two different species. The summary of quantitative and qualitative characteristics of the two species supports those defined in earlier taxonomic work (Clark 1923; Dartnall 1971; Clark and Courtman-Stock 1976; O'Loughlin and Waters 2004) with differences between the two being gonopore position, abactinal surface texture and abactinal surface spinulation. However, SIMPER results also suggest that spinulation on the oral plate, actinal intermediate region, oral marginal plate and furrow/ambulucral (in order of importance) plays a major role in the delineation of these species.

The presence of these two species is in contrast with Dunbar's (2007) results, which demonstrated well-defined clades consisting of sequences from both species. As all the results in this current study maintain this delineation, it is suggested that Dunbar (2007) may have misidentified specimens (no dissections were carried out to determine gonopore position). This is understandable, especially in the case of large *P. exigua* that look morphologically very similar to *P. dyscrita* (personal observation), an example of which is the specimen FL2 seen above. Based on the position of the gonopore, and several other characteristics, it becomes apparent that although FL2 is definitely an individual of *P. exigua*, it is one of the largest specimens collected overall, with spinulation characteristics similar to those of *P. dyscrita*. As a result, it is suggested that some characteristics exhibited by *P. dyscrita* may be the result of this species large size, or the environment in which it lives. Although the former suggestion cannot be tested (little is known about *P. dyscrita*, and even less is known about its juvenile form), FL2 was collected from the front of Strandfontein rockpool, which could be considered a subtidal location. Such results highlight the morphological variability exhibited by *P. exigua* that often seems to be tied to location or environment (an example of which is the Langebaan specimens).

A major limitation of this study is that the sequences obtained from the genetic analyses were not compared to those analyzed by Waters and Roy (2004), or other molecular *P. exigua* studies. This was due to time constraints and may have shed further light on the possibility of cryptic species in South Africa. Kommetjie, where the suggested presence of cryptic species was revealed by Dunbar (2007) could have been exclusively sampled, with all genetic analyses conducted on specimens from this location in order to definitively determine whether a cryptic species occurs at this site or not. Finally, although all work was done by the author, bias could have been introduced when dissected specimens were examined to determine gonopore position, as it could sometimes prove quite difficult (especially in the absence of any visible gonopore position) to distinguish between gonoducts and connective tissue.

Future research should focus on the biology of *P. dyscrita*, of which little is known as well as what drives the morphological variation seen in *P. exigua* specimens found along the different coasts and in sandflat systems. Currently, ecological investigations into both species (and their interaction) are lacking (Dunbar 2007).

Although neither a cryptic species, nor *P. exigua* specimens with aboral gonopores were obtained in this study, we tentatively refute the claim of the presence of aboral gonopores in the South African *P. exigua* population. The distinction between *P. exigua* and *P. dyscrita* is also confirmed, with features that separate these two sympatric *Parvulastra* species clarified.

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Appendix

Table 1A: The primers, DNA dilution, PCR cycle and volume of DNA added according to each specimen that resulted in a successful sequence.

Specimen	Forward Primer	Reverse Primer	DNA Dilution	PCR cycles	Vol. DNA
IS5	LCO1490	Pexig_R1	Straight	38	2
J3	LCO1490	HCO2198	2/10	38	1
IS4	Pexig_F1	Pexig_R1	1/10	35	1
IS6	Pexig_F1	Pexig_R1	1/10	35	1
IS1	LCO1490	Pexig_R1	Straight	38	2
IS3	LCO1490	Pexig_R1	Straight	38	2
K3	LCO1490	HCO2198	2/10	38	1
K2	LCO1490	HCO2198	2/10	38	1
EU1	LCO1490	HCO2198	2/10	38	1
H1	LCO1490	HCO2198	2/10	38	1
K1	LCO1490	HCO2198	2/10	38	1
FL1	LCO1490	Pexig_R1	Straight	38	2
AL30	LCO1490	Pexig_R1	Straight	38	2
CM1	LCO1490	Pexig_R1	Straight	38	2
CH1	LCO1490	Pexig_R1	Straight	38	2
GH4	LCO1490	Pexig_R1	Straight	38	2
CM14	LCO1490	Pexig_R1	2/10	35	1
CM29	LCO1490	Pexig_R1	2/10	35	1
GL1	LCO1490	Pexig_R1	2/10	35	1
GM20	LCO1490	Pexig_R1	Straight	38	2
CL6	LCO1490	Pexig_R1	Straight	38	2
GH8	LCO1490	Pexig_R1	Straight	38	2
CM7	LCO1490	Pexig_R1	Straight	38	2
GM18	LCO1490	HCO2198	2/10	38	1
CM11	LCO1490	Pexig_R1	Straight	38	2
CH23	LCO1490	Pexig_R1	Straight	38	2
AH27	LCO1490	HCO2198	2/10	38	1
BL19	LCO1490	Pexig_R1	Straight	38	2
AH17	LCO1490	HCO2198	2/10	38	1
BL28	LCO1490	Pexig_R1	Straight	38	2
BM19	LCO1490	HCO2198	2/10	38	1
BH8	LCO1490	Pexig_R1	Straight	38	2
BL26	LCO1490	Pexig_R1	Straight	38	2
CL26	LCO1490	HCO2198	2/10	38	1
AM24	LCO1490	Pexig_R1	Straight	38	2
GH27	LCO1490	Pexig_R1	Straight	38	2
CM8	LCO1490	Pexig_R1	Straight	38	2
CH16	LCO1490	Pexig_R1	Straight	38	2
BH16	LCO1490	Pexig_R1	Straight	38	2

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CM10	LCO1490	Pexig_R1	Straight	38	2
AH9	LCO1490	HCO2198	2/10	38	1
GH6	LCO1490	Pexig_R1	Straight	38	2
GH30	LCO1490	Pexig_R1	Straight	38	2
BM4	LCO1490	HCO2198	2/10	38	1
FL2	LCO1490	Pexig_R1	Straight	38	2

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